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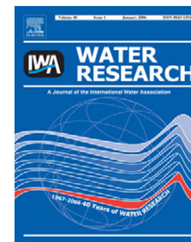

Reactivation and growth of non-culturable indicator bacteria in anaerobically digested biosolids after centrifuge dewatering

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FC, fecal coliform
VBNC, viable but non-culturable
CFU, colony-forming units
MPN, most probable number
DS, dry solids

ABSTRACT

Recent literature has reported that high concentrations of indicator bacteria such as fecal coliforms (FCs) were measured in anaerobically digested sludges immediately after dewatering even though low concentrations were measured prior to dewatering. This research hypothesized that the indicator bacteria can enter a non-culturable state during digestion, and are reactivated during centrifuge dewatering. Reactivation is defined as restoration of culturability. To examine this hypothesis, a quantitative polymerase chain reaction (qPCR) method was developed to enumerate *Escherichia coli*, a member of the FC group, during different phases of digestion and dewatering. For thermophilic digestion, the density of *E. coli* measured by qPCR could be five orders of magnitude greater than the density measured by standard culturing methods (SCMs), which is indicative of non-culturable bacteria. For mesophilic digestion, qPCR enumerated up to about one order of magnitude more *E. coli* than the SCMs. After centrifuge dewatering, the non-culturable organisms could be reactivated such that they are enumerated by SCMs, and the conditions in the cake allowed rapid growth of FCs and *E. coli* during cake storage.

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1. Introduction

During the last few years, researchers have reported relatively low concentrations of fecal coliforms (FCs) after anaerobic digestion; however, immediately after mechanical dewatering, relatively high concentrations of FCs were measured (Iranpour et al., 2003; Cheung et al., 2003; Monteleone et al., 2004; Erdal et al., 2003, 2004; Qi et al., 2004). For example,

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Iranpour et al. (2003) reported results from different field trials using thermophilic digestion. They found that in trials with continuous flow digestion at 57.7 °C as well as a two stage batch digestion at 54 °C, the FCs after digestion were generally below 1000 most probable number (MPN) per gram of dry solids (DSs) with average values near 10^2 MPN/g DS, which meets the United States Environmental Protection Agency (USEPA) requirements for Class A biosolids (USEPA, 1999). However, after dewatering using a full-scale high solids centrifuge and placement in a silo, the FCs were 10^6 MPN/g DS, a dramatic increase of greater than three orders of magnitude. Similarly, Erdal et al. (2003) reported that after mesophilic anaerobic digestion, the FC densities were about 5.6×10^4 colony-forming units (CFU) per gram DS. However, after high solids centrifugation and further conveyance, the FC densities in the cake were 6.5×10^5 cfu/g DS. Storage of this cake for one day resulted in a further increase to 1.5×10^7 cfu/g DS. Similar results after high solids centrifugation have been reported in other studies (Cheung et al., 2003; Qi et al., 2004; Monteleone et al., 2004).

Several different reasons for the large increase in FC density after dewatering have been presented. For example, Qi et al. (2004) suggested regrowth of the FCs was a possible mechanism to explain this phenomena. Alternatively, Cheung et al. (2003) reported that the difference in enumeration between the liquid and the cake was likely due to sample matrix effects. Similarly, Monteleone et al. (2004) suggested that the shear experienced by the solids during high solids centrifugation improved the "release" of the bacteria from the floc matrix which increased the numbers that could be cultured compared with before dewatering. Iranpour et al. (2003) reported regrowth or that contamination of the biosolids with FC could explain the high counts measured after dewatering and storage.

Several potential problems exist with these possible explanations. For example, for regrowth to occur, a significant time is needed to increase the counts by several orders of magnitude. Since the doubling time under ideal conditions for *E. coli* is around 20 min (about the retention time in high solids centrifuges), the large increase in FCs or *E. coli* cannot be explained by regrowth alone. The release of *E. coli* or other FCs from the floc during centrifugation also seems unlikely since during conditioning and dewatering, coagulants such as cationic polymer are added, which aggregate the floc, and allow for the formation of cake. In addition, during the preparation of cake samples for *E. coli* and/or FC analysis, samples are typically diluted in water and homogenized by processes such as blending or put through a stomacher, which should destroy most flocs and release bacteria. In addition, it is not clear that microbial growth would not occur, even if the bacteria are present within a floc matrix.

The authors of this paper propose an alternative hypothesis to explain these results: that the bacteria can enter a non-culturable state during digestion. This renders the bacteria non-culturable using standard culturing methods (SCMs) even though they are present and considered viable. As a result, these SCMs fail to enumerate the viable bacteria present after digestion. During the dewatering process, the bacteria are reactivated or resuscitated, rendering them culturable again. One possible reason that the bacteria become non-culturable is that they enter what is called a "viable but non-culturable" (VBNC) state. A number of different bacteria have been shown to enter a VBNC state such as *E. coli*, *Salmonella sp., Enterococcus faecalis, Shigella dysenteriae, Vibrio cholerae, and Helicobacter pylori* (Byrd et al., 1991; Reissbrodt et al., 2002; Adams et al., 2003; Mizunoe et al., 1999; Gupte et al., 2003). Although there is some controversy in the literature regarding the VBNC state, most of the evidence seems to support this phenomena.

The bacteria that enter the VBNC state often do so after exposure to environmental stress such as nutrient or substrate deprivation, metals, chlorine, salinity, and low temperatures (Mizunoe et al., 1999; Makino et al., 2000; Grey and Steck, 2001; Rockabrand et al., 1999; Lisle et al., 1998). The conditions present during digestion could impart stress that induces the bacteria to enter a VBNC state. These stresses could include low substrate and nutrient concentrations. In addition, for thermophilic digestion, the higher temperatures may also contribute, since higher temperatures have been shown to increase the entrance of certain bacteria into the VBNC state (Adams et al., 2003).

Although the cells enter the VBNC state, they are still considered viable by definition. There has been conflicting evidence in the literature whether the VBNC bacteria can cause infection, but a number of studies have shown that VBNC organisms are capable of causing infection in vivo both in humans and mice (Colwell et al., 1985, 1996; Pommepuy et al., 1996; Cappelier et al., 1999; Chaveerach et al., 2003). In addition, the cells can typically be induced to grow on media in the presence of certain growth promoters or enrichments, and this process is called "resuscitation" or "reactivation" (Lleò et al., 1998; Rockabrand et al., 1999; Makino et al., 2000; Reissbrodt et al., 2000, 2002). The ability of the microbes to be resuscitated is also another indicator of their viability.

The second hypothesis of this research is that dewatering, especially centrifugation, "resuscitates" the VBNC bacteria allowing them to grow and be enumerated using SCMs. The term 'resuscitation' has been used in the microbiology field to describe the transition from non-culturable to culturable. This term has been used interchangeably with "reactivation".

The implications of these hypotheses are significant since much of the design parameters that have been developed to achieve required pathogen or indicator organism destruction are based on using SCMs. Therefore, if these hypotheses are correct, the SCMs may significantly underestimate the actual viable counts of these bacteria.

2. Objectives

The objectives of this research were to examine the following two proposed hypotheses developed to explain field results that found high concentrations of FC and/or *E. coli* after dewatering despite low counts immediately before dewatering:

(1) During digestion, FC and/or *E. coli* can enter a non-culturable state, and are therefore not correctly enumerated by SCMs;
(2) Non-culturable FC and/or *E. coli* can be reactivated during dewatering such that they are able to be enumerated by SCMs.

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